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Biologically-Based Self-Assembling Hydrogels

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ABSTRACT

We have developed polymers, which borrowing from biology, assemble into networks. The self-assembly regions of fibrinogen were cloned to form a scaffolding that either interacts with fibrin or assembles independently. Peptides consisting of a binding pocket (BP), ligand (L), and/or a Factor XIIIa substrate were synthesized and conjugated to methacrylated dextran or acrylated poly(ethylene glycol). Peptide-conjugated dextran was added to polymerizing fibrin, and the resulting hydrogels were evaluated rheologically. These conjugates significantly affected the mechanical properties of fibrin while the addition of unconjugated dextran did not. The BP and L peptides were conjugated to PEG star polymer. Mixtures of conjugated PEG-BP and PEG-L were found to assemble. This work shows that peptides directing assembly can be designed using motifs found in proteins. The peptides in this study not only alter the mechanical properties of fibrin, but also allow a mechanism for creating a self-assembling network.

INTRODUCTION

As material scientists, we stand to learn a lot from nature as biological molecules form precise structures and geometries. With respect to proteins, the primary structure defines the overall three-dimensional architecture. The resulting secondary, tertiary and, in some cases, quaternary structures are necessary to affect intermolecular interactions and protein function. However, examples exist that suggest that short peptides also can be used to affect biological activity including self-assembly.

Material assembly via peptide motifs has been demonstrated. Petka et al. showed how recombinant coiled-coils flanking a random coil polyelectrolyte induced gelation [1]. Gelation was based on the self-assembly of the coiled-coil regions at a controlled pH or temperature. Wang et al. used coiled-coil homotetramers containing histidine tails, copolymers of methacrylic acid, acrylamide, and N-isopropyl acrylamide, and Ni^{2+} to form gels. By tailoring the amino acid sequence of the coiled-coil, they could control the gel response to temperature and pH [2].

We have taken another approach. Our goal was to use biological ligand and receptor interactions to guide the assembly of synthetic polymers. By synthesizing short peptide sequences from fibrinogen that are active in the assembly of fibrin from fibrinogen, we have developed molecules that either can assemble with fibrin or can self-assemble to form three-dimensional complexes.

Fibrinogen is a plasma glycoprotein that participates in the coagulation cascade. The mature protein consists of a dimer of two identical subunits composed of α , β and γ associated chains; three chains associate through a coiled-coil trimer to form a subunit, and two of these subunits associate to form the mature dimer. The assembly of the three chains [3-4], the mature fibrinogen [5], and polymerization of fibrin from fibrinogen [6-8] have been well characterized.

The crystal structure and essential amino acids of the binding pocket for the α -chain-ligand also have been characterized. Oxela and Budzynski found that amino acids 373-410 from

the γ -chain inhibited fibrin polymerization by entrapping the α -chain-ligand; this γ -chain peptide is sufficient to form the binding pocket for the α -chain with a $k_D = 1.45 \times 10^{-6}$ M for the peptide versus 1×10^{-4} M for the intact molecule [9]. Horwitz et al. studied a shorter peptide consisting of amino acids 374-396 of the γ -chain and found that this domain does not contain the platelet binding peptide. In addition, this shorter peptide bound to the N-terminal ligand of the α -chain and was sufficient for formation of the binding pocket [7].

Lorand et al. synthesized a double-headed glycine-proline-arginine-proline, (part of the α -chain ligand) PEG molecule; they found that this molecule induced dimerization of the D proteolytic fragment, which contains the binding pocket, of fibrinogen [10]. This result indicates that PEG molecules containing this tetrapeptide will associate with the binding pocket.

We describe peptide-conjugated polymers that contain blocks of ligand peptide or binding-pocket peptide. These polymers assemble with ligand and binding pocket on fibrinogen or with ligand and binding pocket peptides coupled to other synthetic polymers. Figure 1 shows a schematic of the proposed artificial protein assembly.

MATERIALS AND METHODS

Methacroylation of dextran

Methacroylated dextran was synthesized using published methods [11]. Dextran (MW 40kD) and dimethylaminopyridine (Sigma) were dissolved in dimethylsulfoxide (Sigma) under nitrogen at room temperature. Glycidyl methacrylate (GMA) (Sigma) was added to the mixture to produce GMA-derivatized dextran (dex-GMA). The product was purified by solvent removal and size exclusion chromatography. Aqueous solutions of methacroylated dextran were frozen, lyophilized, and stored at -80°C . The degree of substitution of the purified product was determined by NMR.

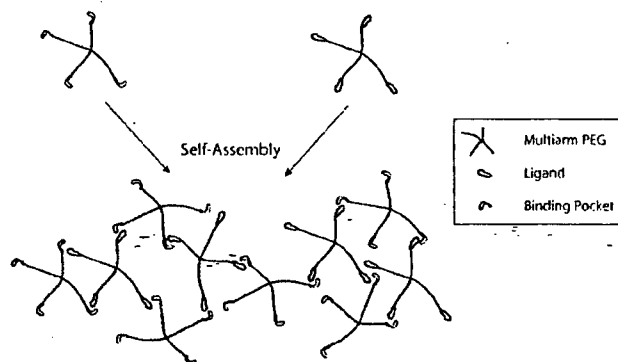


Figure 1. A schematic representation of self-assembly using peptide motifs found within fibrinogen. Multifunctional polymers, such as 4-arm poly(ethylene glycol) can be covalently coupled to ligand or binding pocket peptides inspired by motifs in fibrinogen. A combination of PEG-ligand and PEG-binding pocket will form a three-dimensional physical gel that self-assembles in a manner that mimics fibrinogen to fibrin assembly.

Peptide synthesis

Peptides encoding the binding pocket (BP) from the γ -chain and the ligand from the α -chain of human fibrinogen as well as substrates for factor XIIIa (Fa XIIIa) (Table I) were synthesized using solid-state Fmoc chemistry in the Arizona State University Protein Chemistry Laboratory. All peptides were purified with a C4 reverse phase preparatory column on an ÄTKA FPLC using a water/acetonitrile gradient. Following elution, the peptides were lyophilized and stored at -80°C . Peptide identity was confirmed using MALDI-TOF mass spectrometry (Vestec PerSeptive Biosystems).

Conjugation of peptides to dextran

The following procedure was used to conjugate cysteine-terminated peptide to methacrylated dextran via Michael addition. In separate reactions, a 10 molar excess of each of these peptides was coupled to methacrylated dextran (31% degree of substitution; 40 kD MW) under the following conditions: A solution of dextran was prepared in phosphate buffered saline, 1.5 mM EDTA, pH 7.4. A 10 molar excess of peptide was added to the dextran to create a 20% (w/v) solution. Due to the solubility of the BP peptide, 6 M urea was added to the buffer to allow coupling of BP to dextran. The conjugation reaction proceeded for 2 hours at 37°C . Then, the reaction mixture was dialyzed in 8,000 MWCO dialysis tubing (Spectrum) against MilliQ purified water for 24 hours to remove urea.

Fibrin gel studies

Bovine fibrinogen (Sigma) was dissolved in deionized water and dialyzed against Tris-buffered saline, pH 7.4. A Bradford assay was used to determine the concentration of the fibrinogen solution. Fibrin gels were made to a final volume of 500 μl , and the final fibrinogen concentration of each gel was 0.6%. BP-conjugated dextran was added to 0.5, 1 or 2%. Dextran-LFaXIIIa and dextran-iLFaXIIIa each were added to a final concentration of 2%. For each gel, CaCl_2 to 2.5 mM, 1 unit thrombin (Sigma), and 6 μg of factor XIIIa (Enzyme Research Laboratories) were added, and the gels polymerized for 15 minutes at 37°C . Control gels consisted of unmodified 0.6% fibrin gels and 0.6% fibrin gels containing 2% methacrylated dextran that had not been conjugated to peptide. Dynamic mechanical properties were measured using a 20 mm acrylic parallel plate geometry on a TA Instruments AR 1000 rheometer under a 1 Pa stress throughout a 0.1-100 rad/s frequency sweep [12-13]. The mechanical properties of four gels of each type were compared to determine statistical significance using $\alpha = 0.05$.

Table I. Fibrinogen derived peptides for assembly.

Peptide Function	Amino Acid Sequence*
Binding Pocket (BP)	CGTATWKPGSTRWYSMKKTTMKIIPFNRLTIGE
Ligand (L)	GPRPGGC
Ligand + FaXIIIa (LFaXIIIa)	EGGGVRGPRPNQEQVSPLPC
Inactive Ligand+FaXIIIa (iLFaXIIIa)	CGNQEQVSPLGPRPEGGVR

* Bold letters represent binding pocket or ligand motifs from human fibrinogen. Italicized letters show an amino acid sequence that acts as a substrate for Fa XIIIa. Underlined amino acids indicate a sequence serving as a protective cap (cleaved by thrombin) for the ligand domain.

Acrylation of poly(ethylene glycol)

Poly(ethylene glycol)-tetraacrylate was prepared from PEG 4-arm star polymer (Shearwater, Inc., $M_w = 10,000$) using published methods [14]. Briefly, 25 g 4-arm PEG was dried by azeotropic distillation in 100 mL toluene for 4 hours using a Dean-Stark trap. After cooling, the PEG was resuspended in 25 mL dry dichloromethane (Aldrich). The mixture was placed in an ice bath, and 2 equiv. of triethylamine (Aldrich) was added. Then, 1.7 equiv. of acryloyl chloride (Aldrich) was added to initiate the reaction. The reaction continued with stirring overnight in the dark under nitrogen. The resulting solution was extracted twice with 0.1 N HCl, and the organic phase was precipitated by dropwise addition to diethyl ether (ECM) in an ice bath. After recovery by filtration, the precipitate was dried *in vacuo* and stored under nitrogen at -80°C . NMR analysis confirmed complete derivatization of the 4-arm PEG.

PEG-peptide studies

The synthetic peptide L was combined with acrylated PEG in phosphate-buffered saline, 1.5 mM EDTA, pH 8.0 in a 4:1 molar ratio of peptide to PEG to yield a 1:1 molar ratio of peptide to acrylate group. The peptide BP was conjugated to acrylated PEG in a similar manner; however, the reaction buffer required 6 M urea (Sigma). Each reaction proceeded for 2 hours at 37°C and then was dialyzed against MilliQ water in 8 kD MWCO membrane to remove any unreacted peptide. PEG-BP and PEG-L were combined to make a 5% (w/v) solution that contained equimolar amounts of the two molecules.

RESULTS

Fibrin gel studies

Dynamic mechanical properties were measured on fibrin gels and fibrin gels containing dextran or peptide-conjugated dextran. Dextran alone did not significantly affect the mechanical properties of fibrin (Figure 2). For all concentrations studied, the addition of BP-conjugated dextran significantly disrupted the integrity of the fibrin gels indicating association of the BP with the ligand in fibrin (Figure 2). Increasing the amount of BP-conjugated dextran decreased the storage modulus, G' , of the gel in a dose-dependent manner. Similarly, at 2% concentration, dex-LFaXIIIa significantly disrupted the integrity of the fibrin gels indicating association of the ligand-conjugated dextran with the binding pocket in fibrin (Figure 2). At 2%, dex-iLFaXIIIa significantly increased G' of the fibrin gels to 160% base value, indicating association of the Fa XIIIa substrate-conjugated dextran with the fibrin (Figure 2). The increase in G' with the addition of FaXIIIa substrate-conjugated dextran is expected based on results by Schense and Hubbell demonstrating that synthetic peptides can be conjugated to fibrin via Fa XIIIa [15]. Since the C-terminal protective cap of iLFaXIIIa cannot be cleaved by thrombin, the ligand domain cannot associate with binding pocket in fibrin. Thus, the Fa XIIIa substrate in dex-iLFaXIIIa serves as the only active domain within the peptide. Although dex-LFaXIIIa contains a Fa XIIIa substrate, the substrate appears not to be crosslinked into the fibrin gel suggesting that the ligand/binding pocket interaction dominates. The lack of Fa XIIIa activity of LFaXIIIa may be due to spatial constraints within the peptide

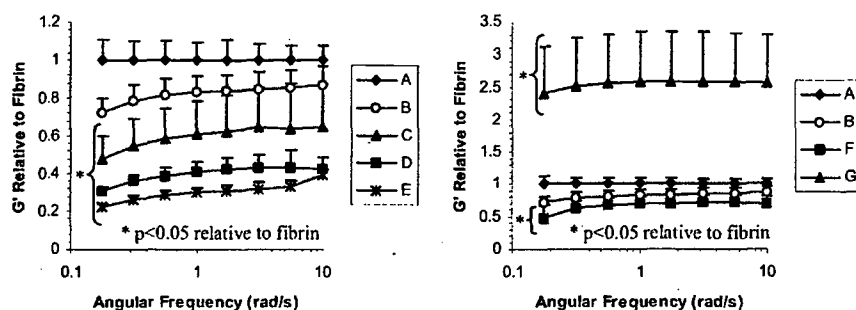


Figure 2. The two plots show the elastic modulus, G' , of the fibrin gels, with or without peptide-conjugate dextran, relative to fibrin as a function of angular frequency for the linear viscoelastic region of 0.6% fibrin gels. For both plots, A=0.6% Fibrin; B=2% Dextran; C=0.5% Dex-BP; D=1% Dex-BP; E=2% Dex-BP; F=2% Dex-LFaXIIIa; G=2% Dex-iLFaXIIIa. As shown in the panel on the left, the BP-conjugated dextran significantly decreased the modulus of fibrin in a dose dependent manner. Dextran alone did not significantly alter G' . In the panel on the right, the dex-LFaXIIIa significantly disrupted the fibrin gel by competitively binding to the native binding pocket in fibrin. The dex-iLFaXIIIa significantly increased the modulus of fibrin through chemical crosslinking via the transglutaminase activity of factor XIIIa.

limiting the ability of the Fa XIIIa substrate to interact with complementary domains within fibrin. These results suggest that proper ratios of ligand-, BP- and Fa XIIIa substrate-conjugated dextran will associate with and affect the mechanical properties of fibrin clots.

PEG-peptide studies

As seen in Figure 3, 5% solutions (w/v) containing only PEG-BP or PEG-L (image not shown) show no evidence of assembly. In contrast, when a 5% solution (w/v) containing equimolar amounts of PEG-BP and PEG-L was observed on a glass microscope slide, the two materials interacted to create a cloudy, viscous suspension. These results suggest a physical self-assembly of the PEG-BP and PEG-L through receptor-ligand interactions. Complete characterization of the association between PEG-BP and PEG-L is ongoing.

CONCLUSIONS

This work provides the first demonstration that peptide sequences directing ligand-receptor interactions in nature can be used to direct assembly of synthetic polymers. We have shown that synthetic polymers can assembly with other synthetic polymers and biologically-derived macromolecules. Future work will involve the optimization of polymer and peptide content to achieve assembly into hydrogels and other useful motifs.

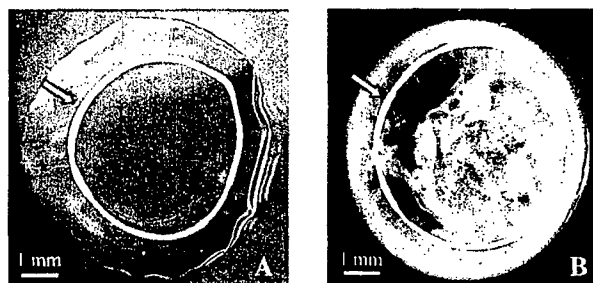


Figure 3. Two stereoscopic images of PEG-peptide solutions on a glass slide. Panel A depicts an image of a 5% PEG-BP solution, and panel B shows an image of a 5% solution containing equimolar amounts of PEG-BP and PEG-L. In both A and B, the white circular rings, denoted by the white arrows, are optical artifacts created by the light source of the stereoscope. As panel B shows, a mixture of PEG-BP and PEG-L results in a physical association.

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